



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁴ : C12N 15/00	A1	(11) International Publication Number: WO 89/12685 (43) International Publication Date: 28 December 1989 (28.12.89)
(21) International Application Number: PCT/US88/02083 (22) International Filing Date: 17 June 1988 (17.06.88) (60) Parent Application or Grant (63) Related by Continuation US 051,366 (CIP) Filed on 18 May 1987 (18.05.87) (71) Applicant (for all designated States except US): INTEGRATED GENETICS, INC. [US/US]; One Mountain Road, Framingham, MA 01701 (US). (72) Inventors; and (75) Inventors/Applicants (for US only) : WYDRO, Robert, M. [US/US]; 28 Raymond Street, Framingham, MA 01701 (US). OPPENHEIMER, Catherine, L. [US/US]; 22 Trinity Drive, Milford, MA 01757 (US). BARNGROVER, Debra, A. [US/US]; 163 Cochituate Road, Wayland, MA 01778 (US). FERRARA, Dawn, T. [US/US]; 5 Stonehedge Road, Lincoln, MA 01773 (US). DACKOWSKI, William, R. [US/US]; 4 Valentine Road, Hopkinton, MA 01748 (US).		LANDES, Gregory, M. [US/US]; 19 Indian Meadow Drive, Northboro, MA 01532 (US). CAPALUCCI, Nancy, A. [US/US]; 283 Trailside Way, Apartment 3C, Ashland, MA 01721 (US). (74) Agent: HOFER, Mark, A.; Integrated Genetics, Inc., One Mountain Road, Framingham, MA 01701 (US). (81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US. Published <i>With international search report.</i>
(54) Title: IMPROVED PROTEIN C MOLECULES AND METHOD FOR MAKING AND ACTIVATING SAME (57) Abstract <p>A method of producing recombinant human Protein C, the method including providing a vector including a DNA sequence encoding human Protein C under the transcriptional control of an eukaryotic metallothionein promoter, the vector further including at least the 69 % transforming region of the bovine papilloma virus; transfecting host eukaryotic cells with the vector; adhering the transfected cells to carrier particles; culturing the carrier particle-bound cells in culture medium to produce the recombinant Protein C; and isolating the recombinant Protein C from the culture medium or the cells. The Protein C is ideally activated by contacting same with thrombin bound to Sepharose beads in low salt concentration. Protein C produced by C127 cells following the above method are unique in that they possess an α-1-3 galactose linkage embueing the protein with longer <i>in vivo</i> half-life.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	ML	Mali
AU	Australia	FR	France	MR	Mauritania
BB	Barbados	GA	Gabon	MW	Malawi
BE	Belgium	GB	United Kingdom	NL	Netherlands
BF	Burkina Fasso	HU	Hungary	NO	Norway
BG	Bulgaria	IT	Italy	RO	Romania
BJ	Benin	JP	Japan	SD	Sudan
BR	Brazil	KP	Democratic People's Republic of Korea	SE	Sweden
CF	Central African Republic	KR	Republic of Korea	SN	Senegal
CG	Congo	LJ	Liechtenstein	SU	Soviet Union
CH	Switzerland	LK	Sri Lanka	TD	Chad
CM	Cameroon	LU	Luxembourg	TG	Togo
DE	Germany, Federal Republic of	MC	Monaco	US	United States of America
DK	Denmark	MG	Madagascar		
ES	Spain				

-1-

IMPROVED PROTEIN C MOLECULES AND METHOD FOR MAKING AND ACTIVATING
SAME

Background of the Invention

5 This invention relates to Protein C and the use of recombinant DNA
techniques to produce Protein C.

10 The coagulation cascade is a series of reactions, involving a number
of plasma proteins, which occur in response to an injury. One of
these plasma proteins, Protein C, helps ensure that the two
important mechanisms in the blood system, clot formation and clot
lysis, are properly balanced so that the body neither bleeds for
undue periods nor clots in places other than the specific site of
15 the injury.

Protein C, in its activated form, inactivates two cofactors in the
blood clotting pathway, factors Va and VIIIa. Protein C has
considerable amino acid sequence homology to other vitamin
20 K-dependent coagulation factors, including prothrombin and factors
VII, IX, and X (Foster et al., 1985 Proc. Natl. Acad. Sci.
82:4673). Protein C, factor VII, factor IX, factor X, and
Prothrombin, are also similar in that each is modified
post-translationally by having the glutamic acid residues in the
25 gamma-carboxyglutamic acid domain of the protein gamma-carboxylated
by specific enzymes, normally present in the liver. These proteins
are inactive without such modification. For example, regarding the
transforming of mouse fibroblast Ltk-cells with the gene encoding
human factor IX (Anson et al. (1985) Nature 315:683), the authors
30 state:

"One cell line was characterized in detail and secreted
detectable human Factor IX in an enzyme-linked
immunosorbent assay, but the secreted factor IX failed to
35 absorb to barium sulphate... Thus, the expression plasmid

was proved functional in producing a protein. However, as expected, the mouse fibroblast cell line lacked the enzymes required for [gamma]-carboxylation of the N- terminal glutamyl residues of factor IX, which are necessary for biological activity."

The authors report that, when the same plasmid was transformed into the rat hepatoma cell line H4-11-E-C3, gamma-carboxylated functional factor IX was produced.

The gene encoding human Protein C has been isolated, cloned, and sequenced (Foster et al., 1984 Proc. Nat. Acad. Sci. 81:4766; Foster et al., 1985 Proc. Nat. Acad. Sci. 82:4673; Beckmann et al., 1985 Nac. Acad. Res. 13:5233). The active protein has been expressed in a human hepatoma (HEPG-2) cell line (Little et al., 1985 Xth Intl. Congress on Thrombosis and Haemostasis, Abstract 0989), and a chinese hamster ovary (CHO) cell line (Little et al., 1985 Xth Intl. Congress on Thrombosis and Haemostasis, Abstract 0990). In CHO cells, transient expression levels were reported to be approximately $1.2 \mu\text{g}/2 \times 10^5$ cells/24h, with 43% of the protein being active.

Activation of Protein C zymogen in vitro by thrombin has been described in the literature for over 10 years (Biochemistry 16: 5824-5831, 1977) However, little attention has been paid to the experimental conditions for this activation. Some authors perform activation in relatively low ionic strength (J. Clin. Invest. 64: 761-769, 1979) while others, probably responding to literature that indicated thrombin activity was stimulated by salt (Arch. Biochem. Biophys. 202: 63-75, 1980), increased the ionic strength to 100-150 mM NaCl (J. Biol. Chem. 258: 8531-8534, 1983). The salt conditions were not changed to distinguish between soluble thrombin, thrombin bound to Sepharose beads (Thrombosis Research 45: 413-419, 1987) or thrombin in complex with thrombomodulin, a thrombin receptor found on endothelial cells (J. Clin. Invest. 73: 968-972, 1984). In addition, recombinant Protein C zymogen activation by snake venom

extracts recently described (Thrombosis Research 47: 85-91, 1987) resulted in activities half that of low salt thrombin activation. It is an object of the present invention to provide improved methods of activation.

5

Summary of the Invention

10

In general, the invention features a method of producing recombinant human Protein C by providing a vector comprising a DNA sequence encoding human Protein C under the transcriptional control of a eukaryotic (preferably mammalian) metallothionein promoter, the vector further comprising at least the 69% transforming region of the bovine papilloma virus, transfecting host eukaryotic cells with the vector, adhering the transfected cells to carrier particles, culturing the carrier particle-bound cells in culture medium to produce recombinant Protein C, and isolating the recombinant Protein C from the culture medium or the cells.

15

20

The above aspect of the invention is based on our discovery that the expression system employed, in combination with microcarrier culturing, produces Protein C of substantially greater biological activity than that produced by the same expression system in tissue culture. Coupled with this are improved methods for activating the zymogen Protein C to the activated form of Protein C.

25

30

Preferred mammalian cells are rodent, e.g., rat or mouse cells, e.g., mouse C127 cells, mouse myeloma cells, and rat hepatoma cells. C127 cells are particularly advantageous in that they are easily transfected and cultured, and can maintain Protein C-containing plasmids in high copy number (over 100 copies per cell) without selection.

35

Preferred vectors of the invention contain DNA (genomic or cDNA) and at least the 69% transforming region, and more preferably all, of

the bovine papilloma virus ("BPV") genome (described in Howley et al., U.S. Patent No. 4,419,446, hereby incorporated by reference). Preferably, there is located downstream from the Protein C-encoding DNA an SV40 DNA fragment, preferably a polyadenylation site.

As will be described in greater detail below, the Protein C made by the recombinant cells in one embodiment of the invention differs from the naturally occurring molecule in that it has an arginine residue at position -4, a modification which has shown some improvement in processing and increase expression in mammalian cells. Another preferred embodiment of the present invention coupled this modification with the substitution of glutamine for arginine at -5 position.

As used herein, "recombinant Protein C" refers to inactive Protein C precursors as well as activated forms of the enzyme. Protein C is synthesized intracellularly in a single chain form and is subsequently cleaved in vivo at four sites. The first cleavage, between a glycine and a threonine residue, results in the removal of the signal peptide necessary for transport into intracellular compartments. The second cleavage, between an arginine and alanine residue, results in the removal of the "pro-peptide" piece necessary for protein recognition by the gamma-carboxylase system. The third cleavage, between an arginine and a lysine residue, results in a heavy and a light chain linked by a disulfide bond. The fourth cleavage, which forms the fully activated enzyme, clips a twelve-amino acid segment from the N-terminal end of the heavy chain. Activation of the enzyme may result in an additional cleavage as well, although whether this is the case or not is at present unknown. This additional cleavage in the clipping of amino acids 156 (lysine) and 157 (arginine), a cleavage which would render the carboxy terminal amino acid leucine, as in mature two chain bovine Protein C. For the sake of simplicity, Fig. 3 is drawn to indicate the presence of amino acids 156 and 157.

We have found that, in the case of some recombinant Protein C we have produced (designated "new" Protein C; see below), the Protein C present in purified fractions has undergone the fourth cleavage, but not the third, forming a biologically active two-chain molecule structurally distinct from naturally occurring active Protein C.

The invention provides biologically active, gamma-carboxylated Protein C useful in medical applications, as will be described in greater detail below. Preferably, the recombinant Protein C of the invention is greater than 95%, more preferably greater than 98%, by weight, pure.

We discovered that full activation of Protein C zymogen is ideally obtained by thrombin bound to Sepharose beads only under low ionic conditions. Unexpectedly, this activity was twice that observed under the traditionally accepted conditions of 100-150 mM NaCl.

Other features and advantages of the invention will become apparent from the following description of the preferred embodiments and from the claims.

Brief Description of the Drawings

Figure 1 is a diagrammatic representation of two human Protein C cDNA clones;

Figure 2 is the 5' terminal sequence of one of said the clones;

Figure 3 is a diagram illustrating the processing of recombinant Protein C;

Figures 4 and 5 are diagrammatic representations of the construction of the intermediate vector ProC-5 and BPV expression vectors; and

Figure 6 is a graphical representation of the relationship between Protein C concentration and clotting time; and

Figure 7 compares snake venom with thrombin-Sepharose bead activation.

Detailed Description and Best Mode

General Approach

In the procedure described below, a cDNA sequence encoding a portion of human Protein C was first isolated from a clone which hybridized to an oligonucleotide prepared to a portion of mature Protein C. A fragment derived from this clone was then used as a second probe to identify a clone containing a cDNA sequence encoding the complete protein. In order to facilitate its insertion into expression vectors, the complete cDNA was modified by in vitro mutagenesis to remove an unspliced intron at the 5' end. These steps are now described in detail.

cDNA for Protein C

Referring to Fig. 1, pA123 is the plasmid which was processed for insertion into an expression vector for expression of Protein C in mammalian cells. Plasmid pA123 contains the entire Protein C coding region (the thickened line, Fig. 1) and also contains an N-terminal intron (which was removed as will be described below). Also shown in Fig. 1 is pA46, a plasmid containing a cDNA which supplied a 350 base pair (bp) probe which was used, as will be described below, to obtain pA123.

The sequence between the translational start point (ATG(-42)) and the first amino acid residue (Ala(1)) of the mature protein represents the "pre-pro" sequence. Referring to Fig. 3, during

processing, Protein C is translated starting at amino acid -42. We have found that signal peptidase cleaves the single chain molecule between residues -25 and -24. This first cleavage removes the "signal" peptide or "pre-"peptide. A second processing cleavage then occurs, by an unknown mechanism, between residues -1 and 1 resulting in a single chain Protein C that starts with the Ala of the mature light chain. Further processing results in Protein C being cleaved so as to produce a non-naturally occurring active species, designated "new" Protein C.

Two polyadenylation and processing sites are shown in Fig. 1 at the 3' end as Poly(A). There is a unique StuI site near the 5' end of the gene (shown in Fig. 2) upstream from a second ATG start site. This second start site may represent the start of a 38 amino acid polypeptide, and is encoded by a portion of an unspliced intron in the cDNA for the Protein C gene.

pA46 is a plasmid clone, derived from a human liver cDNA library, using an oligonucleotide probe made to amino acids 64-77 of the Protein C sequence. Since the 5' end of the Protein C gene was lacking in pA46, its 5' 350 base pair Pst-I fragment was used to reprobe the cDNA library to obtain the complete cDNA clone pA123.

Derivatives of pA123 were constructed, as described below, to facilitate the insertion of the cDNA for Protein C (ProC) into expression vectors, e.g., ProC-5 (Fig. 6), similar to a precursor ProC-3 (Fig. 4) except that the sequence near the ATG start codon of the cDNA has been modified to provide an XhoI site, and the DNA upstream from the modified sequence has been deleted, removing the intron of the cDNA clone.

Adult Liver cDNA Library

A cDNA library may be constructed by any conventional method, such as for example as described by Michelson et al. (1983, Proc. Nat.

Acad. Sci. 80:472). The method used herein involved homogenizing human liver in the presence of guanidine hydrochloride, and then isolating poly(A)⁺RNA by two passages through oligo(dT)-cellulose. cDNA was then synthesized from the liver mRNA in the presence of placental RNase inhibitor (RNasin, Biotec, Madison, WI). cDNA greater than 400 nucleotides long was isolated by alkaline sucrose gradient centrifugation and second-strand synthesis carried out in the presence of the Klenow fragment of Escherichia coli DNA polymerase I (Boehringer Mannheim). The resulting double-stranded cDNA was treated with S1 nuclease (Sigma) and a size fraction of duplex DNA greater than 400 base pairs (bp) in length obtained by sedimentation through a neutral sucrose gradient. Homopolymer tracts of dC were added to the 3' ends of this cDNA and the dC-tailed cDNA then hybridized to Pst I cleaved and dG-tailed plasmid vector pKT218 (Talmadge et al., 1980, Gene 12: 235), and tetracycline-resistant colonies selected after transformation of E. coli strain MC1061 (Michelson et al., 1983, Proc. Natl. Acad. Sci. USA 80: 472). Approximately 120,000 independent recombinant clones were obtained from the original plates, pooled, and stored at -70°C as glycerol stock without further amplification.

Isolation of pA46 and pA123 from the cDNA Library

An oligonucleotide probe was made complementary to the sequence encoding amino acids 64-77 of Protein C using a Standard solid-phase phosphotriester method. The probe had the sequence:
5' TGAAGCTGCCGATGCCGTCGATGCACGTGCACGTGCCCTTG 3'.

In order to identify colonies with DNA homologous to the oligonucleotide probe the colonies were screened on nitrocellulose filters (Millipore HAHY) using ³²P-labeled oligonucleotides. Filters were prehybridized and hybridized as described by Woods et al. (1982 Proc. Nat. Acad. Sci. 79:5661), except that the incubation temperature was 40°C. A clone which hybridized to this probe, pA46, was characterized by restriction enzyme digestion and DNA sequence

analysis and found to contain all of the sequence given in Foster et al., *id.*, plus 42 bp at the 5' end (Fig. 1). Using the PstI cloning site and an internal PstI site, a 350 bp fragment was isolated containing the 5'-most sequence (Fig. 1). This fragment was labeled by nick-translation and used as a probe for screening the adult human liver cDNA library described above. Approximately 120,000 clones were screened with this probe and several positive clones isolated. The majority of the clones isolated were identical to pA46, but one, pA123, was significantly larger, containing the complete coding sequence for Protein C plus a 243bp 5' untranslated sequence and a 60 bp 3' untranslated sequence, and including only the first polyadenylation site in the 3' untranslated sequence.

Construction of ProC-3

In order to insert Protein C cDNA into expression vectors, unique restriction enzyme sites had to be engineered onto the 5' and 3' ends of the Protein C clone. The restriction enzyme site, XhoI, is very rare in nature and is not present in the Protein C coding sequence. This restriction enzyme has been used previously to insert genes into vectors containing the transforming BPV virus. Due to the nature and distribution of the restriction enzyme sites in the Protein C cDNA, a multiple step construction was necessary to engineer the XhoI sites onto the ends. The procedure used is illustrated in Figure 4, and was carried out as follows. pA123 was digested with StuI/AvaI; the StuI site is unique in the Protein C cDNA sequence and is located 164 bp upstream of the ATG in the 5' untranslated sequence. The AvaI site is located in the coding region of the cDNA, about 250 bp upstream from the TAG termination codon (Fig. 1). This StuI/AvaI fragment was purified by electroelution from an agarose gel slice.

pA123 was also digested with PstI and the 880 bp fragment isolated. This 800 bp fragment contains the PstI site within the coding region, 516 bp downstream from the ATG start codon, and the PstI

site 10 bp downstream of the TAG termination codon in the 3' untranslated region. The PstI-PstI fragment was cloned into the PstI site of the plasmid vector pUC 9. The random clones of the chimeric pUC9 were isolated and tested for the orientation of the insert. In one isolated clone, ProC-1, the 5' PstI site of the PstI fragment was located near the EcoRI site in the plasmid vector. This clone was digested with SmaI and then AvaI, mixed with the StuI-AvaI fragment, and ligated. The blunt AvaI ends ligated together, as did the blunt StuI-SmaI ends. The resulting chimeric plasmid, ProC-2, contains the reconstituted coding sequence for Protein C bounded by unique EcoRI and HindIII restriction enzyme sites. For the site directed mutagenesis described in the next section, the Protein C coding region in ProC-2 was excised with HindIII/EcoRI restriction enzymes and cloned into the M13 vector, mpl8. This clone is referred to as mProC-2. Digestion of this plasmid with HindIII/EcoRI, filling in the ends with E. coli polymerase I, and ligating XhoI linkers to the DNA results in a molecule that could be cloned into a pUC9 vector whose SmaI site has been changed to an XhoI site. The resulting plasmid, ProC-3, was grown in bulk culture and provides a source of the XhoI-Protein C-XhoI DNA fragment. ProC-3 was sequenced around the XhoI sites and the AvaI site to confirm that the sequence was not inadvertently altered during manipulations. The sequence analysis showed that no such changes had occurred.

Construction of ProC-5

The strategy for constructing ProC-5 is diagrammed in Figs. 4 and 5. The 5' end of the Protein C cDNA was altered utilizing a synthetic oligonucleotide (oligo B) (this is underscored in the sequence shown in Figure 2). The sequence of this oligonucleotide contains two base pair mismatches to the native Protein C cDNA sequence (noted by asterisks) which provide an XhoI restriction enzyme site immediately 5' of the initiator methionine of the Protein C cDNA. This site, in conjunction with a XhoI site

constructed at the 3' end of the cDNA clone, ProC-3, provided the entire Protein C coding region on a single XhoI fragment suitable for cloning into BPV expression vectors. To accomplish this mutation, the EcoRI-HindIII insert fragment of ProC-2 was first
5 subcloned into the M13 phage vector mpl8 (the resulting clone is termed mProC-2 to distinguish it from the pUC9 ProC-2 clone; see Fig. 4). The single-stranded form of mProC-2 was then isolated and the base pairs changed by site directed mutagenesis using Oligo B, as described below. The mutation was identified by hybridization
10 analysis and a clone (termed m5'XhoProc-2) isolated in bulk quantities.

In order to construct an XhoI site in mProC-2, 5 μ l of single stranded mProC-2, (100-200ng/ μ l) was annealed with 5 μ l of
15 kinased Oligo B (see below, 60ng/ μ l) in 17 μ l double distilled water and 3 μ l of 0.2M Tris, pH7.4, 0.1M MgCl₂, 0.5M NaCl, and 10mM DTT. The mixture was incubated for 6 minutes at 65°C, 60 minutes at 37°C, 10 minutes at room temperature, and then at 0°C until the next step.

The second strand of the phage DNA was synthesized by adding 50 μ l of 30mM Tris, pH 7.5, 10mM MgCl₂, 2mM beta-mercapto ethanol, 10 μ l of 10mM ATP, 3 μ l of E. coli polymerase (klenow fragment) 5 μ l of T4 DNA Ligase (2 u/ μ l), 2 μ l of [alpha-³²P]dATP, and 1 μ l each
25 of 100mM dATP, dGTP, dCTP, dTTP. After incubation for 15 minutes at room temperature the mixture was left at 16°C overnight.

In order to reduce the background of vector which has not been made double stranded by the priming and extension reaction, 70 μ l, of
30 the above extension reaction was mixed with 70 μ l of 0.3M NaCl, 50mM ZnCl₂, 100mM Tris, pH 7.5, 7 μ l S1 nuclease (0.5 u/ μ l), and 553 μ l of double distilled water, incubated at 37°C for one minute, and then at 65°C for ten minutes.

35 ml of this S1 treated extension was then transformed into 300 ml of competent JM101 (*E. coli* cells). As a control, 2 ml of the extension reaction, untreated by S1, was also transformed into JM101.

5 Mutants were identified using filter lifts on the plates containing the plaques from the S1 treated extension reaction. Oligo B was kinased to a high specific activity with ^{32}P -ATP and used as a hybridization probe in 750mM NaCl, 150mM Tris pH 8.2, 10mM EDTA, 5X Denhardt's solution, 0.1% sodium pyrophosphate, 0.1% SDS, and 10 50 $\mu\text{g}/\mu\text{l}$ *E. coli* tRNA. The filters were prehybridized at 37°C for 4 hours. Hybridization was carried out under similar conditions, but with 1.5×10^5 cpm/ml of kinased oligo B, overnight at 37°C. After hybridization, the filters were washed at 15 42°C, 50°C, 54°C, and finally at 57°C. Each wash was in 0.5X SSC for 30 minutes. The resulting filters were autoradiographed for 3 hours, the autoradiograph aligned to the culture plate, and 24 potentially positive (i.e. showing some hybridization) plaques were picked and grown overnight in 5 mls of medium. Plasmid minipreps were performed on the cell pellets and mutants confirmed by 20 digestion with XhoI/HindIII.

The m5'XhoProC-2 construct was ligated to the constructed 3'-Xho end of ProC-3 by splicing the two fragments together at a unique NaeI site. The resulting pUC9 plasmid, ProC-4, was isolated and 25 characterized. A routine part of the analysis of new vectors normally performed is to determine whether all restriction enzyme sites used in constructions are properly regenerated. In this case, the NaeI site used in the construction of ProC-4 was not regenerated. To correct this problem, a BglII-HindIII fragment of 30 ProC-4 was replaced by the same fragment from ProC-3, as shown in Fig. 5a. The NaeI site is contained on the fragment from ProC-3. The final clone, ProC-5, was checked by both restriction map analysis and DNA sequencing to confirm its integrity.

35

Construction of ProC-Gln-Arg

Comparison of the conserved propeptide sequences of vitamin K-dependent proteins indicates that Protein C is the only one without an arginine residue at position -4 in the propeptide sequence. Bentley et al. (1986, Cell 45:343) have proposed that the absence of an arginine residue at position -4 in the propeptide of factor IX results in the production of an unstable profactor intermediate. Thus, in order to promote the proper processing of pro-Protein C (i.e. cleavage at +1 in mammalian cells), the isoleucine residue at position -4 in Protein C was changed to arginine by site direction mutagenesis, as described below. Further, since this mutation would result in a highly arginine rich sequence, the arginine residue at position -5 was changed to glutamine, again by site directed mutagenesis. (PreProthrombin, a highly secreted protein, has glutamine in position -5). These changes are shown below:

Amino acid position:	-5	-4	-3	-2	-1
wild type Protein C:	arg	iso	arg	lys	arg
ProC-Gln-Arg :	gln	arg	arg	lys	arg

These altered proteins should not only be processed better than wild type protein, but should also permit more efficient γ -carboxylation of the mature protein.

The strategy used to construct ProC-Gln-Arg is diagrammed in Fig. 5c. The propeptide end of Protein C cDNA was altered using a synthetic oligonucleotide (oligo C) based on the original DNA sequence. The sequence of oligo C is 3'GG GTG GTT GAC GTC GCG GCG TTT GCA CGG 5'. This sequence contains 3 base pair mismatches to native Protein C cDNA, the sequence of which is shown in Fig. 2 and numbered 341-372. This sequence codes for two amino acid changes in Protein C, one from isoleucine to arginine at position -4, and the other from arginine to glutamine at position -5. To accomplish this

mutation, the 1.4kb EcoRI-HindIII fragment of ProC-5 was isolated and ligated to EcoRI-HindIII treated mpl8, to produce mProC-5. The single stranded form of mProC-5 was isolated and the base pairs changed by site directed mutagenesis using oligo C and the method described above for the construction of ProC-5, i.e. treatment with Klenow and T4 DNA ligase. The mutation was identified by hybridization analysis and a clone (termed ProC-Gln-Arg) isolated in bulk quantities.

Insertion of ProC into Expression Vectors

The modified cDNAs can be inserted into any suitable mammalian expression vector. Preferred expression vectors are the BPV vectors described in Wei et al. U.S.S.N. 782,686, filed October 1, 1985, assigned to the same assignee, and hereby incorporated by reference; and Hsuing et al. 1984, J. Molec and App. Genet. 2: 497. The vectors include a mouse metallothionein promoter from which inserted genes can be transcribed, and bovine papilloma virus DNA to effect transfection of mammalian cells. CLH3axBPV (Fig. 4b) also includes late promoter polyadenylation sequences derived from SV40, which enhance expression of Protein C-encoding cDNA and act as transcriptional stop sequences. The illustrated expression plasmids also include a portion of the E. coli plasmid pML, which permits shuttling between procaryotic and eukaryotic systems. No selection is required for the maintenance of these plasmids in host cells, and they are maintained in high (on the order of 100 copies/cell) copy number.

The XhoI-linkered Protein C sequence in ProC-5 was isolated by digestion with XhoI and excision of the insert band from an agarose gel. This fragment was then cloned into the XhoI site of each of the two BPV vectors, as shown in Fig. 5b. These vectors were then transformed into E. coli strain HB101 and grown in bulk culture. The DNA was purified by CsCl banding before transfection into mammalian cells. The 5' to 3' orientation of the Protein C insert

within the expression vectors was checked by six sets of restriction enzyme digestions. The final vector constructs are shown in Fig. 4b. As a matter of convenience, the names of the expression vectors have been shortened to PCMa and PCSb for CL28XhoBPVProC and CLH3axBPVProC, respectively. "PC" is an abbreviation for Protein C, "M" and "S" stand for the source of the polyA addition sequence (metallothionein (M) or SV40 (S)), "a" refers to Protein C cDNA which has had the 5'-intron removed as described herein while "b" represents the unspliced intron. An additional letter, prefacing these vector designations, indicates the cell line into which the DNA's were transfected (i.e., "C" for C127).

The XhoI fragment of ProC-Gln-Arg may also be inserted into these expression vectors, using similar techniques.

Mammalian Epithelial Cell Transfection

On five separate days, two sets of transfections into C127 cells were carried out (five transfections of PCMa, 42-100mm plates; and five transfections of PCSa, 42-100mm plates), as follows:

Mouse C127 epithelial cells (commercially available) were maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal calf serum (containing small amounts of vitamin K, approximately 1 µg/ml), penicillin/streptomycin, and 10 mM glutamine as described in Hsuing et al., id. These cells have epoxide reductase activity and thus are able to recycle and efficiently use vitamin K for γ-carboxylation of the Protein C produced. The rat hepatoma cell line FAO-1 (HPRT⁻, OUA^r), originally isolated from Faza 967 (Killary et al. 38:523, 1984) and previously shown to produce active prothrombin in the presence of vitamin K (Graves et al., 1980, Biochemistry 19:266), can also be used. Other rodent cells having epoxide reductase activity can also be used, e.g., NIH 3T3 cells (ATCC CCL 92). Cells without this activity require large amounts of vitamin K added to the culture

medium, inefficiently γ -carboxylate protein, and produce a large percentage of biologically inactive Protein C. The vitamin K accumulated by these cells may also build up and block other enzymatic systems of the cell.

5 C127 cells were transfected with PCMb in three separate transfections of 10, 15 and 20 μ g, using salmon sperm DNA as carrier by the method described in Wilger et al. 1977, Cell 11:223, as modified by Hsuing et al., id. The day following transfection, 10 the cells were split into culture plates, some of which contained glass coverslips. On days three and four after transfection, the coverslips were removed and stained with Protein C antibodies. Two sets of controls were performed. The first control was a culture of C127 cells transfected with a non-Protein C vector, split onto 15 coverslips. The second control was Protein C transfected cells which, unlike test cells (below), were not permeabilized with NP-40 after formaldehyde fixation. The control cells gave minimal or no immunofluorescence. In contrast, permeabilized Protein C 20 transfected cultures gave clearly visible staining, which probably results from transient expression of the Protein C vector (the day 4 cells stained less intensely than the day 3 cells).

Two weeks after transfection, transfected cell foci were screened with the filter lift assay. Numerous positive foci were identified 25 by this technique. Control cells treated similarly were negative by this assay. Seventy-nine foci, positive by the filter assay, were picked and transferred to T-25 flasks and cultured in medium containing 10 μ g/ml vitamin K. Of these clones, 72 were screened by ELISA analysis of the culture supernatants. The best 31 clones 30 were then passed to T-75 flasks. Twenty-four hour cell counts and production levels were measured on one set of flasks, while duplicate T-75 flasks were maintained and the production levels assayed every 48 hours. Typically, the T-75 flasks contained approximately 4×10^6 cells in 10 mls of culture media. These 35 cells were assayed for a period of 10 days. Table 1, below, shows

the four best producing cells lines, expressed as grams of Protein C antigen secreted per cell per 24 hours. Table 2, below, gives the production of 6 cell lines based on nanograms Protein C antigen per milliliter of culture fluid. As shown, two cell lines, PCM-4e and PCM-6b, produced levels consistently greater than 1 μ g/ml/24 hours (1 mg per liter of culture medium per day).

TABLE 1

Cell Lines Expressing Protein C Antigen

<u>Cell Line</u>	<u>g/cell/24 hours</u>
PCM-4e	1.62 x 10 ⁻¹²
PCM-4b	9.40 x 10 ⁻¹²
PCM-6b	6.10 x 10 ⁻¹²
PCM-4d	5.40 x 10 ⁻¹²

TABLE 2

Cell Lines Expressing Protein C Antigen

<u>Cell Line</u>	<u>ng/ml</u>	
	<u>48 Hours</u>	<u>24 Hours (5 mls)</u>
PCM-4e	2056	2279
PCM-4b	618	291
PCM-4j	1145	1064
PCM-4f	978	NA
PCM-4b	1224	2784
PCM-4d	791	NA

Purification of Recombinant Protein C

A typical purification regime is as follows. Twenty-three liters of serum-free culture medium containing recombinant Protein C is made
5 0.01% with respect to Tween 80. This media is then clarified to remove particles and debris by filtration through a Sartorius Sartobran 0.5 μ m Filtration Cartridge. After filtration, the pH of the media is adjusted to 5.0 by the addition of 1.0M HCl. The media is then applied to a 100 ml (bed volume) by 5.0 cm diameter
10 Q-Sepharose Fast Flow (Pharmacia) column previously equilibrated with buffer containing: 50mM sodium acetate (NaOAc), pH 6.0/50mM NaCl/0.01% Tween 80. The flow rate through the column is not critical but is routinely 20 ml/minute. After application of the culture medium, the column is washed with 8-10 bed volumes of buffer
15 containing: 50 mM sodium acetate (NaOAc), pH 7.0/50mM NaCl/0.01% Tween 80. A step elution buffer of 500mM NaCl 0.01% Tween 50mM NaOAc pH 7.0 is applied to the column and 20 ml fractions collected. The peak Protein C fractions are identified by assaying the column fractions with a commercial Protein C ELISA kit
20 (Diagnostica Stago). Peak fractions are pooled. Recovery of recombinant Protein C from this column are generally 95-100%.

The pooled fraction from the Q-Sepharose column is diluted 1:1 with buffer containing 10 mM Tris, pH8.0/10mM CaCl_2 /0.01% Tween 80.
25 This material is applied to an immunoaffinity column previously equilibrated with buffer containing: 50mM Tris, pH 7.5/150mM NaCl/5mM CaCl_2 /0.05% Tween 80. The immunoaffinity column is prepared by binding a human Protein C specific monoclonal antibody to CNBr activated Sepharose 4B (Pharmacia) by standard protocol at a
30 ratio of 5mg antibody per milliliter of bead. The monoclonal antibody is calcium-dependent, i.e., it binds Protein C only in the presence of calcium ion, the antibody is made by conventional methods. The column diameter is 5cm and the bed volume is determined at a ratio of 1 milliliter of bed volume per milligram of
35 Protein C in the pooled Q-Sepharose fraction. After application of

the recombinant Protein C, the column is washed with 8-10 bed volumes of buffer containing 50mM Tris, pH 7.5/150mM NaCl/5mM CaCl_2 /0.01% Tween 80. The column is then washed with 8-10 bed volumes of buffer containing 50mM Tris, pH 7.5/2.0M NaCl/2mM CaCl_2 /0.01% Tween 80. The recombinant Protein C is eluted from the column with buffer containing 100mM Glycine/0.01% Tween 80 pH 4.0. Column eluate is collected in fractions and neutralized by the addition of one-tenth volume of buffer containing: 1.0M Tris, pH 8.0/0.01% Tween 80. Recovery of recombinant Protein C from this column is typically 80%.

If purity in excess of about 98% is required, the resultant purified Protein C may be further purified by applying the following procedure. The recombinant Protein C from the immunoaffinity column is diluted 1:1 with buffer containing: 100mM NaOAc, pH 6.0/30mM NaCl/0.01% Tween 80. This material is then applied to a 30 ml (bed volume) 2.5 cm diameter DEAE-Trisacryl (LKB) column previously equilibrated with buffer containing: 50mM NaOAc, pH 6.0/15mM NaCl/0.01% Tween 80. After application of the sample, the column is washed with 8-10 bed volumes of buffer containing: 50mM NaOAc, pH 6.0/15mM NaCl/0.01% Tween 80. The recombinant Protein C is then batch eluted with buffer containing: 50mM NaOAc, pH 6.0/750 mM NaCl/0.01% Tween 80. Recovery of recombinant Protein C from this column is generally 95-100%. The resulting recombinant Protein C is 99% pure or greater as judged by the following protocol: 2.0-2.5 μ grams of the purified Protein C is applied to an 8x10x0.08 cm reduced SDS polyacrylamide gel and electrophoresed by standard Laemmli technique. The gel is then stained by standard Coomassie-blue technology. The washed gel is then scanned by laser densitometry and recombinant Protein C and contaminant bands quantified.

The recombinant material contains major proteins that comigrate with the natural light chain and two heavy chain bands of Protein C. In addition to these bands, there is a higher molecular weight band

that potentially corresponds to single-chain Protein C. The amount of material in this band corresponds more closely to the level of Protein C being expressed in the culture medium than to the volume of culture medium passed over the antibody column. A densitometer tracing of a similar gel indicated that approximately 70% of the staining material is in this high molecular weight band.

The purified recombinant Protein C is measured for biological activity by a coagulation assay. This assay was chosen because of its dependence on the gamma-carboxyglutamic acid residues of Protein C. Protein C clot assay protocol is provided in detail later. The standard used is ideally highly purified human Protein C available from Dr. Johan Stenflo (University of Lund, Malmo, Sweden).

Figure 6 shows a typical standard curve and Table 3 give the average clotting times of the standards and the recombinant Protein C samples. Sample #3 was measured both in the presence and absence of Protac C. Without Protac C, the clotting time was indistinguishable from buffer alone, indicating that the sample was activatable by a Protein C activator and that no activated Protein C was present in the preparation. When compared to the ELISA values for the Protein C content of each sample, #3 and #4 were approximately 50% biologically active, whereas #5 was approximately 75% biologically active. The reason for the difference in biological activity of these fractions is not known. It is possible that, since the monoclonal antibody used for purification is Ca^{++} dependent and that one of the Ca^{++} binding regions on Protein C involves the gamma-carboxyglutamic acid residues, the fraction of Protein C not fully gamma-carboxylated exhibits a weaker binding affinity for the column and therefore elutes earlier than the fully gamma-carboxylated molecule. The extent of gamma-carboxylation and biological activity in clotting assays are directly correlated.

TABLE 3

Protein C Dependent Coagulation Test

5

	<u>Sample</u>	<u>Average Clotting Time (sec)</u>	<u>Protein C μg/ml</u>
10	1	37.3	0
	2	58.9	1
	3	79.6	2
	4	95.7	3
	5	105.9	4
15	6	132.4	5
	#3 (1:5)	77.2	9.9 (corrected)
	#3 (1:5)-ProtacC	37.1	0
	#4 (1:5)	86.5	11.3 (corrected)
20	#5 (1:5)	59.9	6.2 (corrected)

Production of Protein C Using Microcarriers

25 Protein C was produced from CPM-4e cells grown on microcarriers as follows:

Microcarrier spinners (Bellco Glass Co.) were washed, air dried, siliconized (Sigmacote, Sigma Chemical Co.), and dried. The
30 spinners were then extensively rinsed with glass-distilled water.

Cytodex 3 (Pharmacia) inert polymeric microcarrier beads were added to each spinner and the spinners were then filled to one-half of final working volume with phosphate buffered saline (PBS). The
35 beads were allowed to swell at room temperature for three hours,

after which they were rinsed twice with PBS by decanting and the spinner volume returned to one-half of final working volume. The spinners were then loosely capped, prepared for autoclaving, and steam sterilized at 121°C for one to two hours. After cooling to room temperature, the PBS was withdrawn to 20% of final working volume and DME medium added to final volume. The resulting culture was stirred for five minutes at 37°C, and the beads then allowed to settle, after which 80% of the supernatant was replaced with fresh DME medium containing 10% fetal bovine serum.

Freshly trypsinized PCM-4e cells from confluent roller bottles were added to the spinner cultures (initial cell concentration = $1 - 2 \times 10^5$ cells/ml). The cultures were then placed on magnetic stirrers (stirring speed = 45 rpm for a one liter vessel) and the cells allowed to grow at 37°C. Starting on day three after seeding, 80% of the growth medium was replaced every two days with fresh medium until cell concentration was $1 - 2 \times 10^6$ cells/ml (cells were enumerated by counting nuclei). The cultures were then put into Protein C production phase by replacing the growth medium with IG-1 serum-free media supplemented with 10 µg/ml of vitamin K₁. The media was then replaced with fresh media every 24 hours. The oxygen overlay of the vessels was maintained at 20%. The samples were assayed for Protein C antigen using the Diagnostica Stago Assera Protein C ELISA Kit. It was found that under these conditions, one liter spinner flasks could be maintained at an average expression level of 6 mg/l/24 hours for 30 days. Omitting vitamin K₁ from the culture medium or increasing oxygen overlay to 40% reduced Protein C production.

The biological activity of samples taken from three Protein C microcarrier productions was determined using the coagulation assay described previously. Although a few samples exhibited low (approximately 30%) biological activity, most samples exhibited close to 100% biological activity and high Gla content (5 moles per mole protein). In contrast, Protein C prepared by the microcarrier

method in the absence of vitamin K₁ exhibited no biological activity and low Gla content. Protein C isolated from PCM-4e - conditioned media from cells grown in tissue culture and not on microcarriers, in the presence of vitamin K, exhibited high Gla content but consistently low biological activity.

Use

Recombinant Protein C of the invention can be used in the treatment of patients with congenital and acquired Protein C deficiencies. Congenital Protein C deficiency, for which there is currently no effective treatment, afflicts one of 16,000 persons, with 75% experiencing recurrent thrombolytic problems starting at age 25 to 35.

Acquired Protein C deficiency occurs in persons with vitamin K deficiency, liver disease, disseminated intravascular coagulation (a condition wherein blood clots form all over the body), and may also occur during post-operative periods. Hip surgery patients, numbering 167,000 per year in the United States, often have reduced Protein C levels for approximately five days following surgery. During this post-operative stage, these patients are at high risk for deep vein thrombosis, and it is expected that restoring Protein C to normal levels will help minimize this risk.

Another therapeutic application of Protein C is as an adjunct therapy to fibrinolytic agents, particularly tissue plasminogen activator (TPA). Co-administration of Protein C and TPA could diminish the TPA levels needed and is therefore expected to lead to a more cost-effective treatment of cardiovascular patients.

Protein C of the invention will be admixed with a pharmaceutically acceptable carrier substance, e.g., saline, and administered orally, intravenously, or by injection into affected tissues. For treatment of Protein C deficiency (defined as less than 50% normal Protein C

in the blood), sufficient Protein C is administered to bring blood levels up to at least 1.5 milliequivalents/ml (3 milliequivalents/ml is normal). Ordinarily, this will require administration of about 10-100 mg, most preferably about 60 mg, of Protein C to an average human adult.

Another potential use of protein C is as an anti-coagulate for blood in vitro.

Currently, drawn blood is anti-coagulated with either sodium citrate or EDTA. Large transfusions of this blood can lead to toxic effects of the sodium citrate or EDTA. Protein C, being a natural substance, would not have these toxic effects.

A second, similar use is in interoperative "recycled" blood. Blood normally lost in abdominal and chest spaces during surgery is suctioned into a machine where it is washed and readministered to the patient. This blood is anti-coagulated with sodium citrate and EDTA, and during extended surgery the patient is susceptible to toxic effects. Using Protein C in the activated form, instead of sodium citrate and EDTA, to anti-coagulate this blood could prevent such toxic effects in the patient.

Deposits

The vector CL28XhoBPVPROC was deposited, on July 24, 1986, in E. coli strains with the American Type Culture Collection (ATCC), Rockville, Maryland, and assigned ATCC accession number 67164.

Unique Protein C Characteristics

It has been surprisingly discovered that Protein C produced in C127 host cells transformed with the vector CL28 XhoBPVROC contains the α -1-3 galactose linkage within the carbohydrate moiety, which linkage is similar to the human blood group B determinant as

demonstrated by its reactivity with human immunoglobulin. This linkage lends additional biological characteristics to the Protein C molecule such as increased in vivo half-life in primates possibly in turn due to an apparently increased immunological reactivity with normally present immunoglobulins. CHO cells transformed with a suitable vector incorporating the same Protein C cDNA sequence do not produce Protein C with the α -1-3 galactose linkage and the accompanying biological advantages.

Protein C Clot Assay Protocol

The following is the protocol currently used to measure the biological activity of the recombinant Protein C.

Materials:

1. Anticlot C Activator (American Diagnostica, Inc.) - This reagent contains PROTAC C, a Protein C activator derived from viper venom, which is co-lyophilized with APTT reagent.
2. Protein C deficient plasma (American Diagnostica, Inc.) - lyophilized.
3. Normal human plasma (American Diagnostica, Inc.) - lyophilized. Protein C antigen has been determined by electroimmunodiffusion (Laurell rocket technique) using a pooled normal human plasma preparation as 100%.
4. Imidazole-Buffered Saline, 10 x concentrate (American Diagnostica, Inc.).
5. Bovine serum albumin (Sigma #A-7638).
6. 0.025 M Calcium chloride solution.

7. Coag-A-Mate XC (Organon Teknika Corporation) - An automated photo-optical coagulation instrument.

5 Assay Protocol:

1. Reconstitute Anticlot C Activator with 1.5 ml purified water.

10 2. Reconstitute Protein C deficient plasma with 1.5 ml purified water. Allow plasma to stand at room temperature for 15 minutes before use for complete reconstitution. Store on ice until use.

15 3. Reconstitute normal human plasma with 0.5 ml purified water. Allow plasma to stand at room temperature for 15 minutes before use for complete reconstitution. Store on ice until use.

4. Warm up instrument and set-up assay parameters on the Coag-A-Mate Xc instrument as follows:

20	Test: APTT	Blank time: 20.0 sec.
	Pump 1 vol: 75 μ l	Activation time: 300.0 sec.
	Pump 2 vol: 75 μ l	Maximum clot detection time: 150.0 sec.

25 5. Prepare buffer by diluting the 10x concentrate with purified water to yield a 1x solution and dissolve BSA to a concentration of 1 mg/ml. This solution may be prepared in advance and stored at 4°C for up to two weeks.

30 6. Prepare plasma Protein C standards from the reconstituted human plasma (putative concentration of 3 μ g Protein C) as follows:

35 - A stock solution containing 600 ng/ml Protein C is prepared by diluting 300 μ l of normal human plasma in 1.2 ml imidazole-BSA buffer.

- The remaining standards are prepared from the stock solution:

5	<u>Concentration</u>	<u>Stock Antigen Volume</u>	<u>Buffer Volume</u>
	0 ng/ml	-	200 µl
	60 ng/ml	50 µl	450 µl
	120 ng/ml	100 µl	400 µl
10	240 ng/ml	200 µl	300 µl
	360 ng/ml	300 µl	200 µl
	480 ng/ml	400 µl	100 µl

7. Prepare the Coag-A-Mate for operation by connecting the Anticlot C activator and calcium chloride reagent reservoirs to the first and second dispensing pumps, respectively. Prime the tubing making certain to eliminate any air bubbles. The tubing should be checked for air bubbles before starting each run and reprimed if necessary.

20

8. Standard/samples are assayed (in duplicate) for clotting time by pipetting the following reagents into appropriate wells of the assay tray:

25 75 µl Protein C deficient plasma
 +75 µl standard or sample

Each assay tray holds twelve sample wells.

9. Place the assay tray into the instrument and start the reaction. The remainder of the assay is performed automatically and follows the following sequence:

35

+75 µl Acticlot C activator
Incubate 5 minutes at 37°C
+75 µl Calcium chloride (0.025 M)
Measure clotting time

10. The concentration of Protein C in the samples is determined from the standards using a curve fitting computer program that calculates the data based on a second order quadratic equation ($[\text{Protein C}] = A \times [\text{clot time}]^2 + B \times [\text{clot time}] + C$).

Correlation coefficients of the data to the equation are generally greater than 0.98, reducing the errors associated with data transformation.

Assay Performance:

The performance of the assay is monitored with a purified Protein C preparation which serves as an internal control and is included in every assay. The inter-assay variability (n=28) currently has a coefficient of variation of approximately 10%. The precision of the assay (n=11) with respect to the internal control has a coefficient of variation <10%. The assay bias is unknown due to the lack of a standard Protein C preparation with a known specific activity.

Protein C Amidolytic Procedure

A. Reagents and Buffers

1. Sample Dilution Buffer:

5X Stock: 0.25 M TrisCl, pH8.0; 0.5 M NaCl; 0.05 M CaCl_2
0.5% BSA. Sterile filter fresh stock, store at 4°C.

Working: Dilute 5X Stock 1:5 with ddwater. Add 4µl of ThromStop for every 100µl of buffer.

2. Imidazole Buffer:

Solution A: 3.03 grams Tris; 1.7 grams imidazole; 50 ml 1N HCl in 100 mls distilled water.

Solution B: 4.04 grams Tris; 2.27 grams imidazole; 1.95 grams NaCl in 100 mls distilled water.

10X Working: Mix solutions A and B to obtain 200 mls of solution pH 8.4 at 25°C. Add to the 200 milliliter solution 23.4 grams NaCl to obtain a concentrate, ionic strength = 3.0. Store at 4°C.

Working Buffer: I-0.3: 1/10 dilution in water of 10X concentrate.

3. ThromStop (American Diagnostica). Take up one bottle (1.0 μ moles) in one milliliters water, store at 4°C for up to three weeks.

4. Spectrozyme PCa (American Diagnostica). Take up one bottle (1.0 μ moles) in 2.5 milliliters water. Store refrigerated for up to three weeks or aliquoted and frozen (no more than once) for several months.

B. Procedure

1. Dilute stock of activated Protein C in Sample Dilution Buffer to make a 5 mU/ml stock.

2. Using the 5 mU/ml stock make a standard curve ranging from 1 to 40 mU/well (assuming 10 μ l will be added per well. Keep on ice until ready to use.

3. Set up a 96-well flexible Falcon plate, by adding to each well:

80 μ l I = 0.3 Imidazole buffer

10 μ l of standard or sample

10 μ l Spectrozyme PCa

4. Incubate at 37°C for 30 minutes

5. Stop the reaction with 20 μ l glacial acetic acid

6. Read absorption at 405 nanometers

Thrombin Bead Assay

Thrombin-Sepharose Beads:

Immobilized thrombin was prepared by coupling purified bovine thrombin (Sigma T7513, 2196 U/mg) to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's directions; ethanolamine was used to block unreacted sites. Quantitative coupling of protein was obtained. Yield of active thrombin averaged 33-40% as determined by the thrombin assay described below. Activity of the coupled beads was in the range of 80-120 U/ml of packed beads.

Amidolytic Assay

Each reaction is done in a 1.5 ml Eppendorf centrifuge tube. Thrombin (Sigma T7513) is diluted to 0.1 U/ml in 50mM Citrate, pH 6.5; 150mM NaCl; 0.01% Tween 80. Bead slurry (1 ml of buffer per 1 ml of packed beads) are generally diluted 1/400. Spectrozyme TH (American Diagnostica) is suspended in 1 ml of double distilled water per vial.

Standard Curve

	Units of <u>Thrombin</u>	<u>Buffer</u>	0.1U/ml <u>Thrombin</u>	<u>ddH₂O</u>	<u>Spectrozyme</u>
5	0	50μl	0μl	200μl	50μl
	1	50μl	10μl	190μl	50μl
	3	50μl	30μl	170μl	50μl
10	6	50μl	60μl	140μl	50μl
	10	50μl	100μl	100μl	50μl
			Beads		
		50μl	25μl	175μl	50μl
		50μl	50μl	150μl	50μl

15

Incubate for 30 minutes at 37°C with agitation. The reaction is quenched by the addition of 20μl glacial acetic acid. Spin beads down one minute in an Eppendorf Microfuge. 250μl is then read in a spectrophotometer at 405nm. The thrombin activity on the beads is then compared to the soluble thrombin.

20

Protein C Activation

25

Either a purified or partially purified Protein C zymogen is dialyzed against 100 volumes of buffer containing 50 mM Tris-HCl (pH 8.3, range = pH 7.0 - 8.5); 1 mM EGTA. Dialysis buffer would be replaced once with fresh buffer to ensure the low salt content of the Protein C sample.

30

Thrombin-Sepharose beads were prepared by coupling 250 units of thrombin (Sigma) per milliliter of wet packed cyanogen bromide activated Sepharose 4B (Pharmacia) as described in the "Handbook of Affinity Chromatography" obtained from the manufacturer (Pharmacia). Thrombin-Sepharose beads were stored in buffer

35

containing 50 mM Na citrate (pH 6.5); 150 mM NaCl and 0.01% Tween 80

at a ratio of 1 ml buffer per ml packed wet beads at 4°C (for long storage periods 0.05% sodium azide is added to the buffer). Units of thrombin activity coupled to the beads were determined using a synthetic chromogenic substrate, Spectrozyme TH (American
5 Diagnostica) as described by the manufacturer of the synthetic substrates. Generally, recovery of thrombin activity on the beads was 30-60%.

10 Activation of Protein C zymogen was advantageously performed at Protein C concentrations of between about 10 and 1000 µg and at thrombin concentrations of between about 1 and 20 units per ml. Monovalent cation concentrations (i.e., Na⁺, K⁺) are ideally kept below 50 mM, incubation was for 2-6 hours at 37°C. Figure 7
15 compares results of two preparations AB8, AB9 (two runs each A and B) activated by snake venom activation and by the thrombin-Sepharose beads of the present invention. The advantages of the invention are readily apparant.

Example of Activation Protocol

20 To a polypropylene screw cap tube was added the following: 200 µl of 5X activation buffer (250 mM Tris-HCl), pH; 8.4, 0.05% Tween 80); 20 µl 50 mM EGTA; 125 µl of 40 U/ml Thrombin-Sepharose slurry (slurry = 1 ml buffer to 1 ml packed beads); 1.0 mg of dialyzed
25 Protein C zymogen; and distilled deionized water to a final volume of 1.0 ml.

The tube was capped tightly and incubated at 37°C for four hours with continuous agitation. After incubation, thrombin beads were
30 removed by centrifugation for five minutes in a clinical centrifuge. The activated Protein C supernatant was decanted and assayed for protein concentration by assay in ELISA (Diagnostica Stago) and Bradford protein assay (Bradford, M.; Anal. Biochem
35 72:248 (1976)) and activity in an amidolytic and clot assay as previously described.

The following results were obtained:

Protein C Activations

	Run 1	Run 2	Run 3	Run 4	Run 5
Natural pC ¹	986±153	446±40	450±47	141±10	1097±312
Days 3 FBS ²	470±85	348±68	225±45	879±113	429±24
Days 3 DCS ³	471±35	345±28	260±74	849±112	468±51

¹ Immunopurified from cryoprecipitated normal pooled plasma

² Purified from conditioned media on day 3 of production in a 50L Marubishi fermenter of C127 cells transformed with the aforementioned deposited vector, fed during growth phase with 10% fetal bovine serum.

³ Purified from conditioned media on day 3 of production in a 50L Marubishi fermenter of C127 cells transformed with the aforementioned deposited vector, fed during growth phase with 10% donor calf serum.

The World Health Organization Standard for Protein C, 86/622, has, by definition, 0.81IU/ml which, taking an average of 3 µg Protein C/ml plasma, is equivalent to 273 IU/mg, in all cases significantly less than the recombinant Protein C of the present invention.

Claims

1. A method for producing recombinant human Protein C having at least 50% of the biological activity of purified Protein C or at least about 100% of the activity of the World Health Organization Standard for Protein C.(86/622), said method comprising:
- a) providing a vector comprising a DNA sequence encoding human Protein C under the transcriptional control of a eukaryotic metallothionein promoter, said vector further comprising at least the 69% transforming region of the bovine papilloma virus;
 - b) transfecting host eukaryotic cells with said vector;
 - c) adhering said transfected cells to carrier particles;
 - d) culturing said carrier particle-bound cells in vitamin K supplemented culture medium to produce said recombinant Protein C; and
 - e) isolating said recombinant Protein C from said culture medium or said cells.
2. The method of Claim 1 wherein said vector includes all of said bovine papilloma virus genome.
3. The method of Claim 1 wherein said carrier particles are inert polymeric beads.
4. The method of Claim 1 wherein said culturing comprises oxygenating said cells at a level less than about 40%.

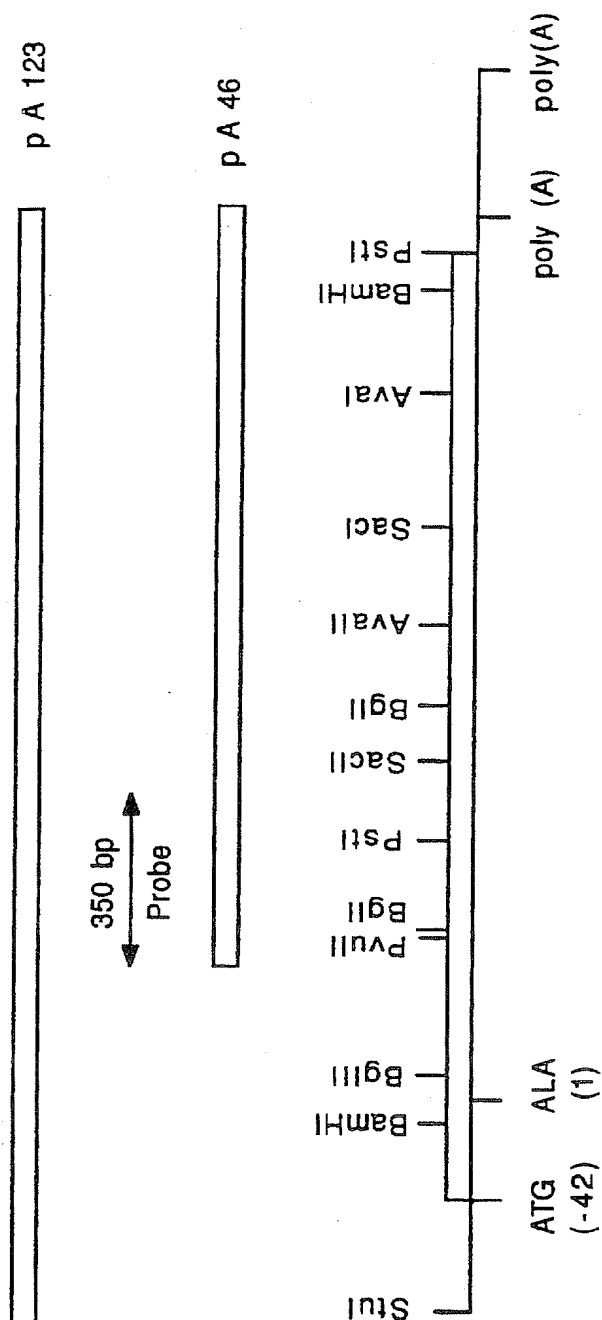
5. The method of Claim 1 wherein said carrier particle-bound cells are agitated during said culturing to keep them in suspension.
- 5 6. The method of Claim 5 wherein said vector includes, between the C-terminal encoding end of said DNA encoding human Protein C and said bovine papilloma virus genome DNA, a fragment of SV40 DNA.
- 10 7. The method of Claim 6 wherein said SV40 fragment comprises a polyadenylation site for expression of said DNA encoding said human Protein C.
- 15 8. The method of Claim 1 further comprising the step of activating said Protein C isolated in step e) with thrombin coupled to beads under conditions where monovalent cation concentrations are less than about 50mM.
- 20 9. The method of Claim 1 wherein said eukaryotic host cells are C127 cells.
10. Recombinant human Protein C expressed by the method of Claim 1.
- 25 11. Recombinant human Protein C expressed by the method of Claim 9.
12. Recombinant ProProtein C having an arginine residue at position -4.
- 30 13. DNA encoding ProProtein C, said DNA encoding an arginine residue at position -4.
14. Recombinant ProProtein C having a glutamine residue at position -5.

35

15. DNA encoding ProProtein C, said DNA encoding a glutamine residue at position -5.
- 5 16. Recombinant human Protein C which is greater than 95% pure, by weight.
17. The recombinant Protein C of Claim 13, said recombinant Protein C being greater than 98% pure, by weight.
- 10 18. The recombinant Protein C of Claim 14, said recombinant Protein C being greater than 98% pure, by weight.
19. The recombinant Protein C produced by the method of Claim 11 containing an α -1-3 galactose linkage.
- 15 20. Recombinant Protein C containing an α -1-3 galactose linkage.
21. The protein of Claim 20 possessing a longer in vivo half-life than natural human Protein C.
- 20 22. Recombinant Protein C having a longer in vivo half life than natural Protein C.

1/9

FIG 1



SUBSTITUTE SHEET

2/9

FIGURE 2

10 30 50
TCTGGAGCTGCTTTCTAGGCAGGCAGTGTGAGCTCAGCCCCACGTAGAGCGGGCAGCCGA

70 90 110
StuI
GGCCTTCTGAGGCTATGTCTCTAGCGAACAAGGACCCTCAATTCCAGCTTCCGCTGACG
MetSerLeuAlaAsnLysAspProGlnPheGlnLeuProProAspG

130 150 170
GTCAGCACACAGGGACAGCCCTTTCATTCCGCTTCCACCTGGGGGTGCAGGCAGAGCAGC
lyGlnHisThrGlyThrAlaLeuSerPheArgPheHisLeuGlyValGlnAlaGluGlnG

190 210 230
Nci I GGAGC
AGCGGGGGTAGGCACTGCCCGGAGCTCAGAAAGTCCTCCTCAGACAGGTGCCAGTGCCCTCC
lnArgGlyEnd

250 270 290
TCCTACACCGTCGAGTGTTT Bal I
AGAATGTGGCAGCTCACAAGCCTCCTGCTGTTCTGTGGCCACCTGGGGAATTTCCGGCACA
MetTrpGlnLeuThrSerLeuLeuLeuPheValAlaThrTrpGlyIleSerGlyThr

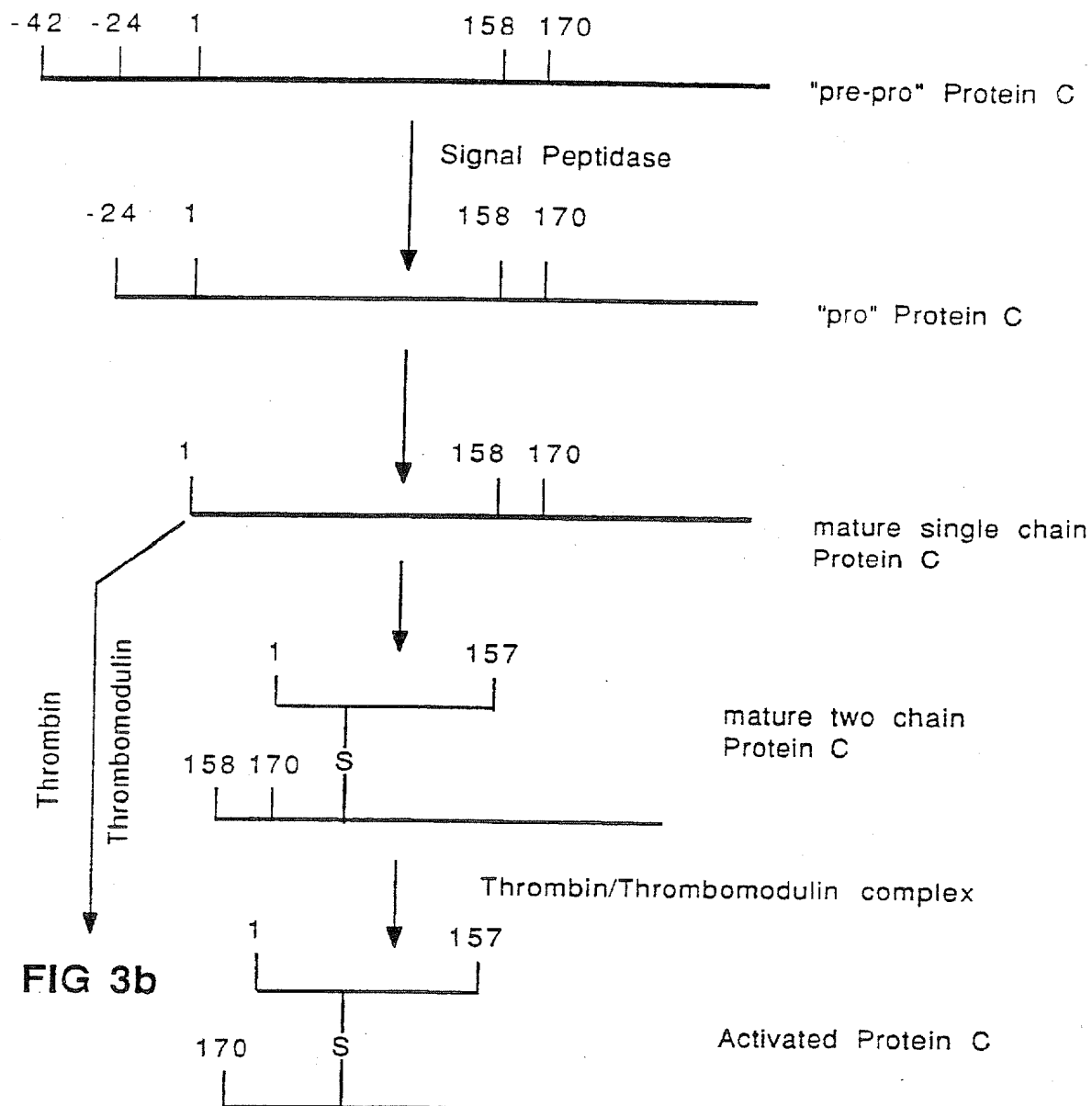
310 330 350
CCAGCTCCTCTTGACTCAGTGTTCTCCAGCAGCGAGCGTGCCCAAGTGCTGCGGATC
ProAlaProLeuAspSerValPheSerSerSerGluArgAlaHisGlnValLeuArgIle

370 390
CGCAAACGTGCCAACTCCTTCTTGAGGAG
ArgLysArgAlaAsnSerPheLeuGluGlu

SUBSTITUTE SHEET

3/9

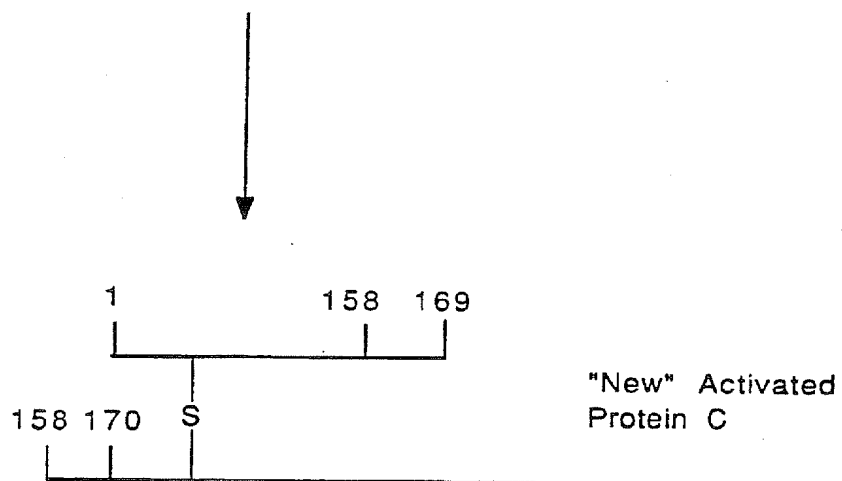
FIG 3a



SUBSTITUTE SHEET

FIG 3b

FIG 3a



5/9

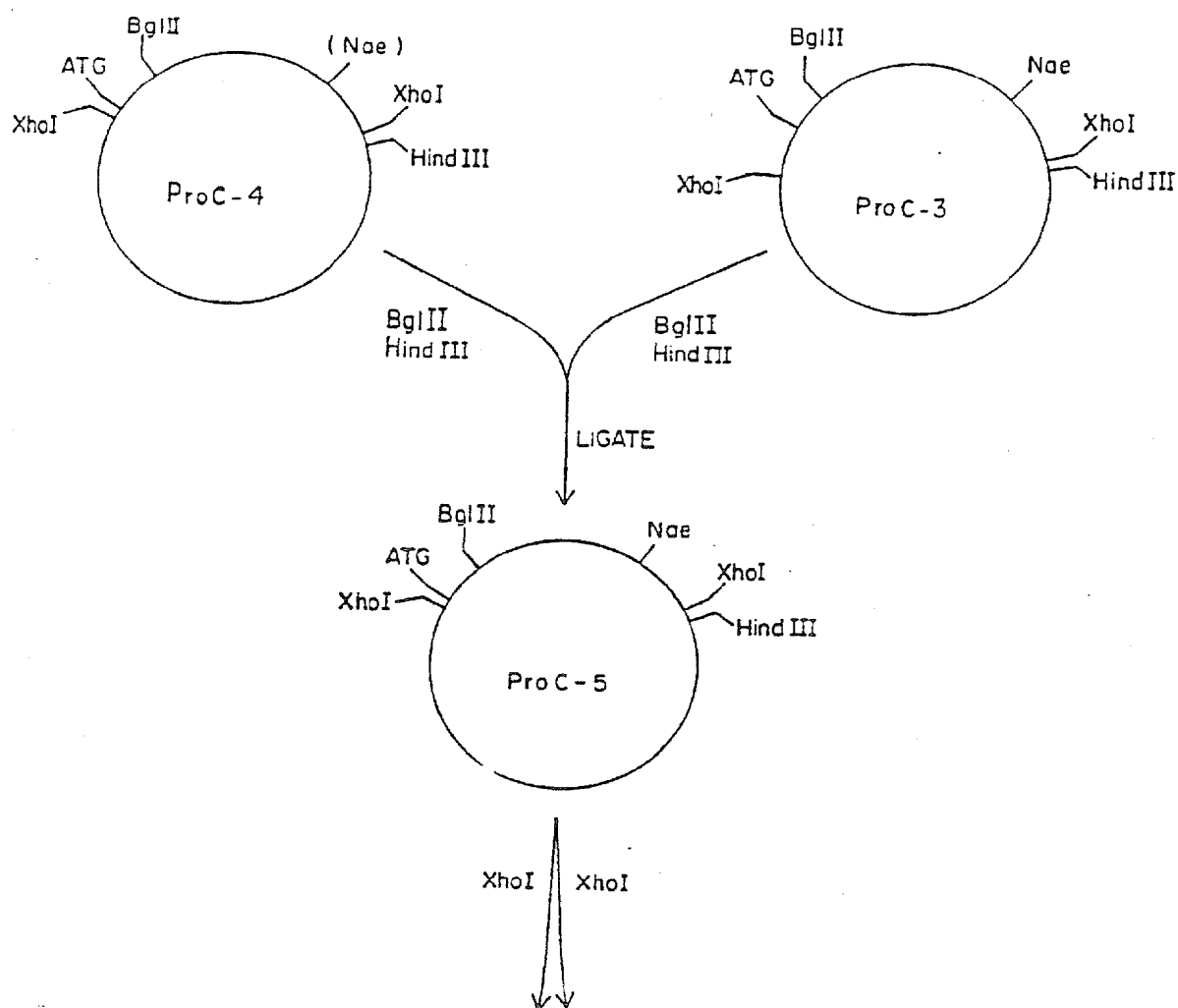


FIG 5a

SUBSTITUTE SHEET

6/9

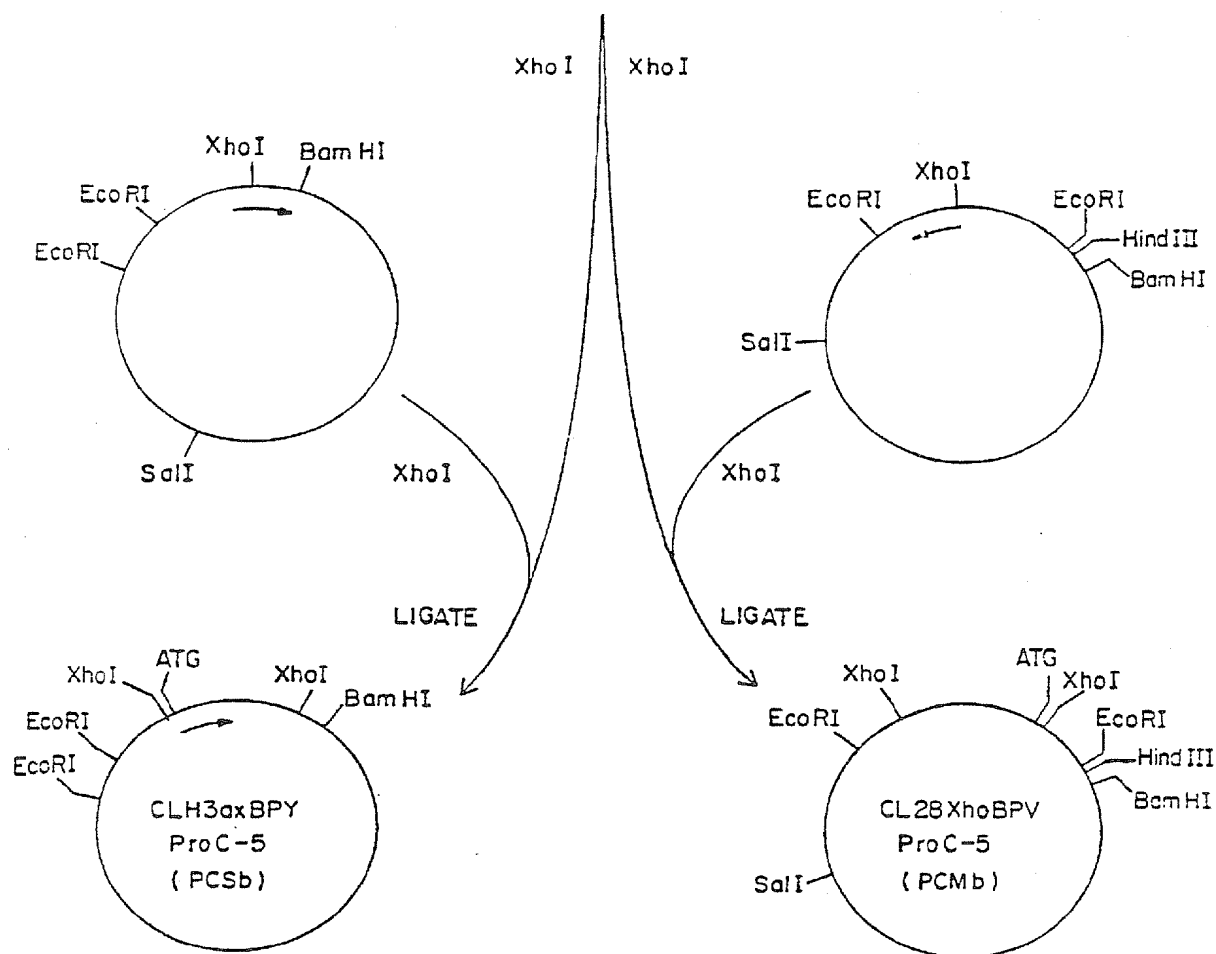


FIG 5b

SUBSTITUTE SHEET

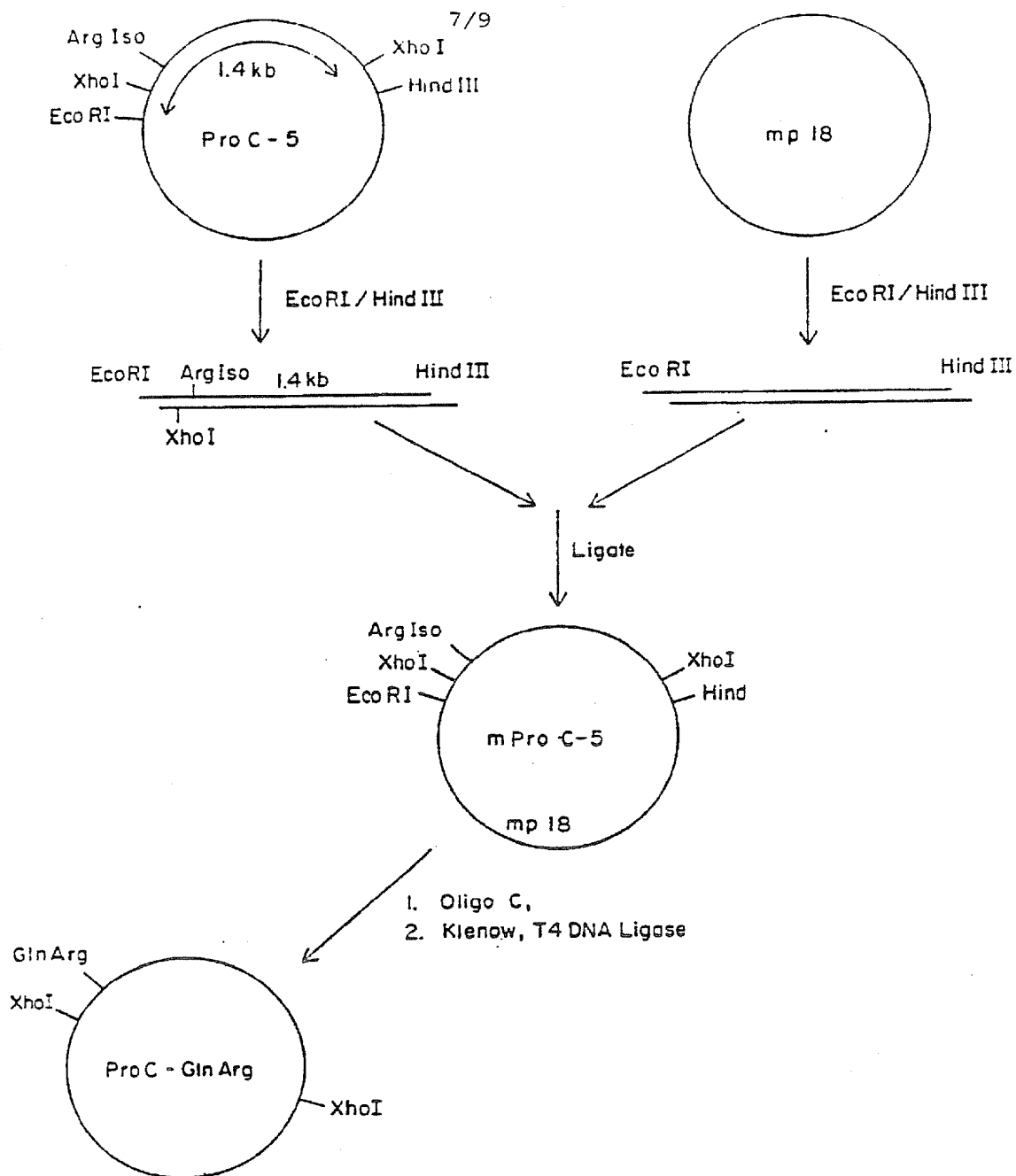


FIG 5c

SUBSTITUTE SHEET

8/9

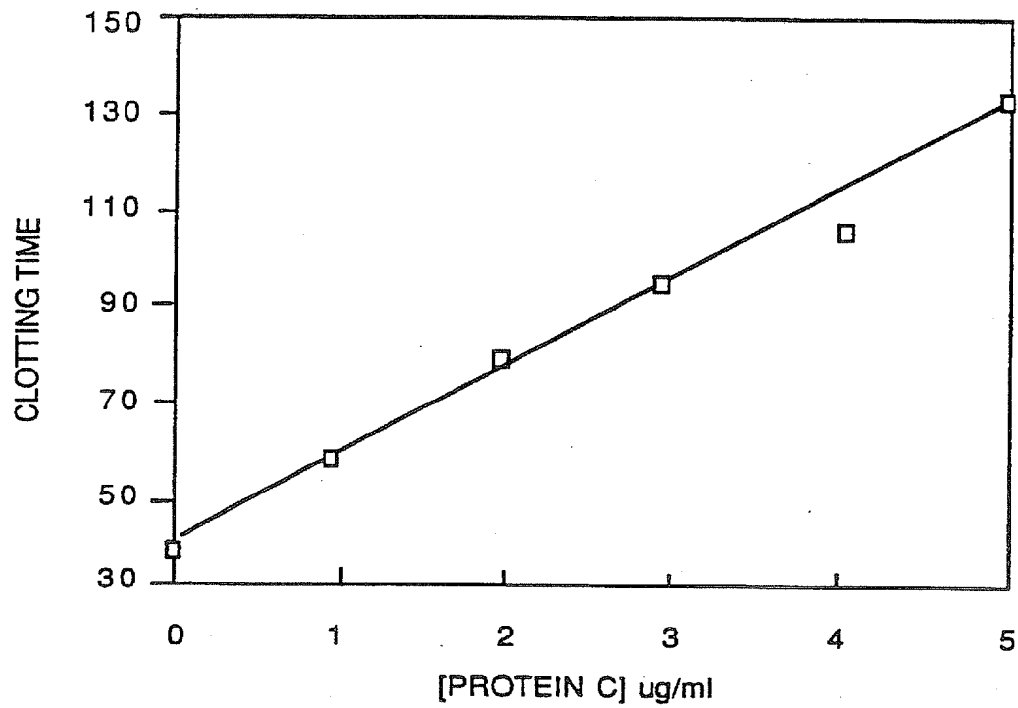
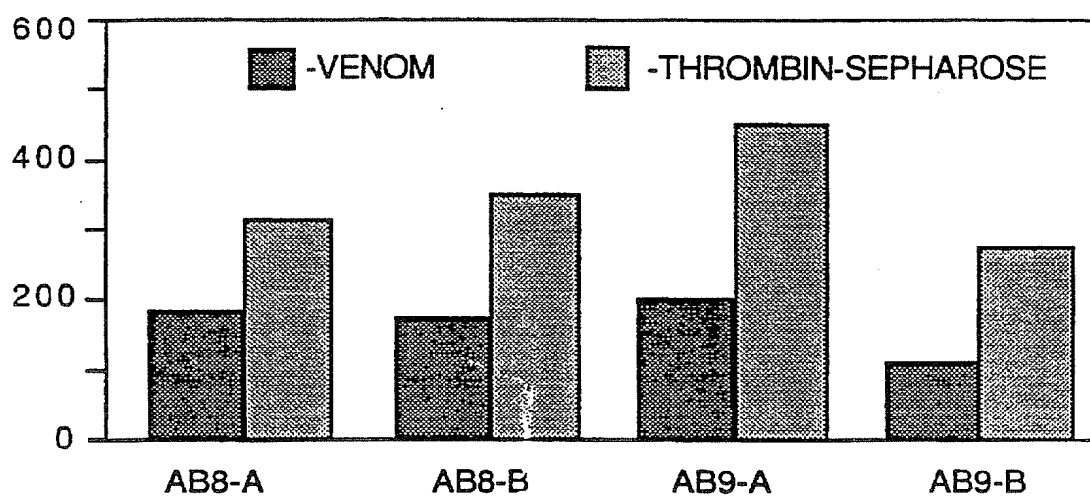
FIG 6**SUBSTITUTE SHEET**

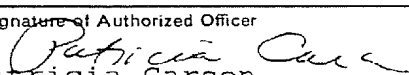
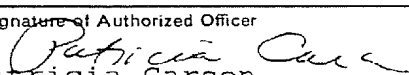
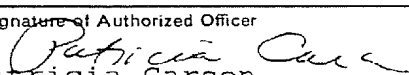
FIG 7

9/9

**SUBSTITUTE SHEET**

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/02083

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC C12N 15/00 U.S. 435/172.3														
II. FIELDS SEARCHED <div style="text-align: center; margin-top: 10px;">Minimum Documentation Searched ⁷</div> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 20%;">Classification System</th> <th style="width: 80%;">Classification Symbols</th> </tr> <tr> <td style="text-align: center; vertical-align: middle;">U.S.</td> <td>435/172.3, 68, 70, 253, 320, 823, 886, 849 935/29, 32</td> </tr> </table> <div style="text-align: center; margin-top: 10px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸</div>			Classification System	Classification Symbols	U.S.	435/172.3, 68, 70, 253, 320, 823, 886, 849 935/29, 32								
Classification System	Classification Symbols													
U.S.	435/172.3, 68, 70, 253, 320, 823, 886, 849 935/29, 32													
Chemical Abstract Data Base (CAS) 1067-1988. BIOSIS DATA BASE 1969-1988 Keywords-human protein C, cloning, plasma protein.														
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%;">Category *</th> <th style="width: 70%;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 20%;">Relevant to Claim No. ¹³</th> </tr> <tr> <td style="text-align: center; vertical-align: top;">X</td> <td>Proc. Natl. Acad. Sci Vol 81 issued August 1984 (Washington D.C.)(Foster et al) characterization of a cDNA coding for human protein C see entire document particularly Figure 2 p. 4766-4770</td> <td style="text-align: center; vertical-align: top;">1-22</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">X</td> <td>Nucleic Acids Research Vol. issued August 1985 (Oxford, England) (Beckmann et al) The structure and evolution of a 461 amino acid human protein C precursor and its messenger RNA based upon the DNA sequence of cloned human liver cDNA's see entire document Particular Figure 35233-5243</td> <td style="text-align: center; vertical-align: top;">1-22</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">X</td> <td>EP 0191606 published 20 August 1986 Elli Lilly and Co. see particular page 21 U.S.A. 4,775,624 (Bang et al) 04 October, 1988 see entire document particularly col. 17 and 18</td> <td style="text-align: center; vertical-align: top;">1-22</td> </tr> </table>			Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	X	Proc. Natl. Acad. Sci Vol 81 issued August 1984 (Washington D.C.)(Foster et al) characterization of a cDNA coding for human protein C see entire document particularly Figure 2 p. 4766-4770	1-22	X	Nucleic Acids Research Vol. issued August 1985 (Oxford, England) (Beckmann et al) The structure and evolution of a 461 amino acid human protein C precursor and its messenger RNA based upon the DNA sequence of cloned human liver cDNA's see entire document Particular Figure 35233-5243	1-22	X	EP 0191606 published 20 August 1986 Elli Lilly and Co. see particular page 21 U.S.A. 4,775,624 (Bang et al) 04 October, 1988 see entire document particularly col. 17 and 18	1-22
Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³												
X	Proc. Natl. Acad. Sci Vol 81 issued August 1984 (Washington D.C.)(Foster et al) characterization of a cDNA coding for human protein C see entire document particularly Figure 2 p. 4766-4770	1-22												
X	Nucleic Acids Research Vol. issued August 1985 (Oxford, England) (Beckmann et al) The structure and evolution of a 461 amino acid human protein C precursor and its messenger RNA based upon the DNA sequence of cloned human liver cDNA's see entire document Particular Figure 35233-5243	1-22												
X	EP 0191606 published 20 August 1986 Elli Lilly and Co. see particular page 21 U.S.A. 4,775,624 (Bang et al) 04 October, 1988 see entire document particularly col. 17 and 18	1-22												
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>														
IV. CERTIFICATION <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; padding: 5px;"> Date of the Actual Completion of the International Search 17 October 1988 </td> <td style="width: 50%; padding: 5px;"> Date of Mailing of this International Search Report <div style="font-size: 1.2em; font-weight: bold;">1 8 NOV 1988</div> </td> </tr> <tr> <td style="width: 50%; padding: 5px;"> International Searching Authority ISA/US </td> <td style="width: 50%; padding: 5px;"> Signature of Authorized Officer <div style="text-align: center;">  Patricia Carson </div> </td> </tr> </table>			Date of the Actual Completion of the International Search 17 October 1988	Date of Mailing of this International Search Report <div style="font-size: 1.2em; font-weight: bold;">1 8 NOV 1988</div>	International Searching Authority ISA/US	Signature of Authorized Officer <div style="text-align: center;">  Patricia Carson </div>								
Date of the Actual Completion of the International Search 17 October 1988	Date of Mailing of this International Search Report <div style="font-size: 1.2em; font-weight: bold;">1 8 NOV 1988</div>													
International Searching Authority ISA/US	Signature of Authorized Officer <div style="text-align: center;">  Patricia Carson </div>													

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, 1 st with indication, where appropriate, of the relevant passages 1 st	Relevant to Claim No 1 st
X	Cell Vol. 45 issued May 1986 (Cambridge, Mass.) (Bentley et al) Defective Pro-peptide processing of Blood clotting factor IX caused by a mutation of Arginine to Glutamine at position-4 pages 343-348.	12-18 and 20-22
Y	U.S.A 4,579,821 (Palmiter et al) 1 April 1986 See abstract, example 1	
Y	USA 4,751,084 (Feder et al) 14 June 1988 See example 1	
Y	EP 0200421 Published 12 October 1986 Zymo-genetics inc. See particularly back-ground	1-22
Y	U.S.A 4,189,534 (Levine et al) 19 February 1980 entire document	1,5
Y	Nucleic Acids Research Vol. 13 issued August 1985 (Oxford England)(Portela et al) A primer vector system that allows temperature dependent gene amplification and expression in mammalian cells regulation of the influenza virus NSI gene expression see abstract, material and methods pages 7959-7968.	6-7
Y	Nucleic Acids Research Vol. 10 issued August 1982 (Oxford England)(Zoller et al) Oligonucleotide- directed mutagenesis using M13 derived Vectors an efficient and general procedure for the production of point mutations in any Fragment of DNA see entire document pages 487-6496.	12-15
Y	Methods in Enzymology Vol.80 issued August 1982. (New York)(Kisiel et al.) Protein C pages 320-322.	1-22

